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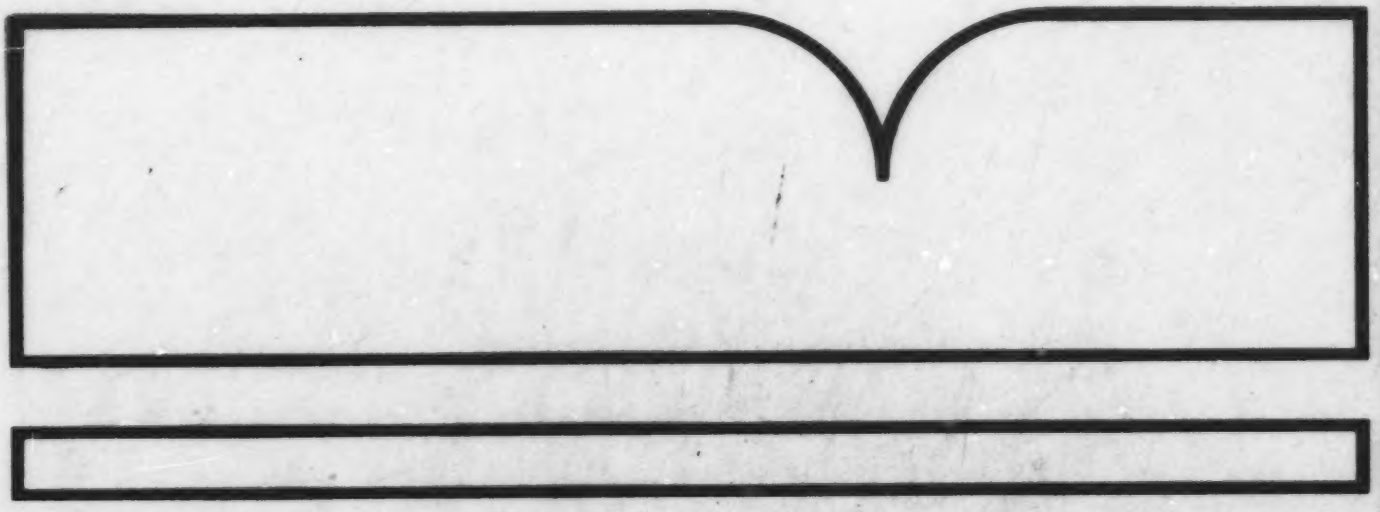

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**16. ABSTRACT** Conceptuses removed from the rats in the eleventh day of gestation were cultured in vitro for 2 days. Growth and differentiation of the major organs of the embryo in vitro resembled those developed in vivo. Embryonic development and organogenesis were markedly affected when the alkylating agents TEM (2,4,6-triethylenimino-1,3,5-triazine) and nitrogen mustard (mechlorethamine hydrochloride) were added to the culture medium. At concentrations of 1 and 5μg/ml medium, these teratogens were highly embryotoxic and affected both growth and differentiation. DNA and protein content of embryos and yolk sacs was reduced significantly ($p<0.001$) from the controls. Development of conceptuses in the culture medium that contained cyclophosphamide (0.35 mM) alone apparently were normal. However, addition of cyclophosphamide (0.35 mM), microsomes (0.5 mg protein/ml), and NADPH (1 mM) to the culture medium induced marked deleterious effects on the conceptus growth and defferentiation. DNA and protein contents were significantly ($p<0.001$) reduced by the combined treatment indicative of formation of reactive metabolites and their interference with macromolecular biosynthesis. Aminopyrine which has not been shown to be teratogenic, in equimolar amounts to cyclophosphamide, under identical conditions of culture, did not affect conceptus development.

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# Rat Conceptus Development *in Vitro*: Comparative Effects of Alkylating Agents

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Rat Conceptus Development *in Vitro*: Comparative Effects of Alkylating Agents. SANYAL, M. K., KITCHIN, K. T., AND DIXON, R. L. (1981). *Toxicol. Appl. Pharmacol.* **57**, 14–19. Conceptuses removed from the rats in the eleventh day of gestation were cultured *in vitro* for 2 days. Growth and differentiation of the major organs of the embryo *in vitro* resembled those developed *in vivo*. Embryonic development and organogenesis were markedly affected when the alkylating agents TEM (2,4,6-triethylenimino-1,3,5-triazine) and nitrogen mustard (mechlorethamine hydrochloride) were added to the culture medium. At concentrations of 1 and 5 μg/ml medium, these teratogens were highly embryotoxic and affected both growth and differentiation. DNA and protein content of embryos and yolk sacs was reduced significantly ($p < 0.001$) from the controls. Development of conceptuses in the culture medium that contained cyclophosphamide (0.35 mM) alone apparently were normal. However, addition of cyclophosphamide (0.35 mM), microsomes (0.5 mg protein/ml), and NADPH (1 mM) to the culture medium induced marked deleterious effects on the conceptus growth and differentiation. DNA and protein contents were significantly ($p < 0.001$) reduced by the combined treatment indicative of formation of reactive metabolites and their interference with macromolecular biosynthesis. Aminopyrine which has not been shown to be teratogenic, in equimolar amounts to cyclophosphamide, under identical conditions of culture, did not affect conceptus development.

It is well known that alkylating agents induce developmental anomalies in the offspring of laboratory animals (Murphy *et al.*, 1958; Chaube and Murphy, 1968). These agents cause retardation of growth and abnormal development of various organs. The ability of teratogens to cause malformation *in vivo* depends upon a large number of variables including the stage of pregnancy when exposure occurs, route of exposure, maternal metabolism and excretion, transplacental transfer, and detoxification and bioactivation processes in both maternal and fetal tissues (Wilson, 1973). In addition, abnormal embryonic development of the embryos may be secondary to maternal toxicosis in the *in vivo* test systems.

The methods for culturing rat embryos *in vitro* have improved considerably in recent years (New, 1973, 1978; Steele, 1975; Sanyal, 1980; Sanyal and Wiebke, 1979). In these systems, conceptuses taken from rats in tenth to twelfth day of gestation consisting of embryos within yolk and amniotic sacs and polar ectoplacental cones are cultured in roller bottles with appropriate oxygen atmospheres. Differentiation and initial morphogenesis of major organs (e.g., brain, sensory organs, heart, circulatory system,

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muscle, and somites) occur *in vitro* in the cultured embryos. The growth and differentiation of these organs *in vitro* are comparable to that *in vivo* during the same periods of gestation (New, 1978; Sanyal, 1980; Sanyal and Wiebke, 1979). The direct effects of teratogens on the embryos and membranes can be studied conveniently in this system.

Preliminary studies show that the indirectly acting alkylating agent, cyclophosphamide, is activated to induce anomalous development by addition of cyclophosphamide, microsomes, and cofactors together to the medium in which conceptuses are cultured (Fantel *et al.*, 1979a,b; Sanyal *et al.*, 1979; Kitchin *et al.*, 1979). In the present study, we compared the effects of direct acting alkylating agents TEM (2,4,6-triethyleniminо-1,3,5-triazine) and nitrogen mustard (mechlorethamine hydrochloride) to the effects of indirectly acting alkylating agent (cyclophosphamide) that was activated by a microsomal activating system. We also studied the effects of a nonteratogen, aminopyrine, on *in vitro* embryo–placental development utilizing the conceptus culture system.

## METHODS

Adult female rats (Charles River CD strain) of 70–80 days age were obtained pregnant, or colonized proestrus females were bred and maintained under normal laboratory conditions of 14 hr of light with rat chow and tap water. Conceptuses of pregnancy Day 11 (sperm positive pregnancy Day 1; embryonic age 10.5 days at noon) with embryos concave in fetal position at Witschi stage 16 containing 15–16 somites were dissected out removing the decidual tissues and exposing the yolk sac (Witschi, 1962). They were cultured for 46–48 hr in rotating 30-ml bottles on horizontal bars according to the methods described earlier (Sanyal, 1980; Sanyal and Wiebke, 1979). They were grown in fresh serum immediately separated from blood obtained from 75- to 80-day-old male rats. Each conceptus was cultured in 5 ml medium at 38°C with 20% $O_2$, 5% $CO_2$, and 75% $N_2$ gas atmosphere for 24 hr and 40% $O_2$, 5% $CO_2$, and 55% $N_2$ for the next 22–24 hr.

Microsomes were isolated from adult male rat liver as described previously (Kitchin and Woods, 1978). Liver tissues from adult animals were homogenized in 1.15% KCl containing 0.02 M Tris–HCl buffer, pH 7.5, and centrifuged at 9000*g*. The microsomal pellet was obtained by centrifugation of 9000*g* supernatant at 105,000*g*. The microsomal pellet was then washed, recentrifuged, and finally suspened in cold serum medium. The microsomal mixed-function oxidase enzyme activities, assessed by aminopyrine *N*-demethylation to formaldehyde (Nash, 1953), in serum medium with 20% $O_2$, 5% $CO_2$, and 75% $N_2$ gas phase in rotating bottles was 83% of microsomes suspended in 0.05 M Tris–HCl buffer, pH 7.5, in air during a 15-min period (7.31 mmol formaldehyde/mg microsomal protein/min).

During the afternoon (1500–1700 hr), conceptuses from several rats (3–4 isolated from each rat) were assigned to different treatment groups to reduce the litter variation. The treatment groups with media containing different constituents are as follows: (a) cyclophosphamide (100 μg/ml, 0.35 mM); (b) cyclophosphamide (0.35 mM), microsomes (0.5 mg protein/ml), and NADPH (1 mM); (c) aminopyrine (80 μg/ml, 0.35 mM); (d) aminopyrine (0.35 mM), microsomes (0.5 mg protein/ml), and NADPH (1 mM); (e) TEM (1 and 5 μg/ml); (f) nitrogen mustard (1 and 5 μg/ml). Control cultures having no additives were performed under identical experimental conditions along with the treatment groups with littermate conceptuses harvested at the same time. Each treatment group was composed of four to eight conceptuses derived from different rats.

At the end of the 46- to 48-hr culture period, conceptuses were removed from the medium and washed in 5 ml Ringer's solution. The yolk sac diameter, beating of the heart, and somite number were noted using a binocular stereomicroscope. Appearance of anatomical anomalies in the embryo, deviation from *in vivo* differentiation, were assessed visually. The embryo, yolk sac, and placenta (basal allantoic placenta) dissected from each conceptus were frozen in liquid nitrogen and stored at −15°C until utilized with 2–3 weeks for DNA and protein measurements. These tissues were homogenized in 1 ml ice cold 0.05 M phosphate buffer, pH 7.4, with a sonifier (Branson Sonic Power Co.). DNA was estimated by ethidium bromide fluorescence after digestion of aliquots of homogenate with RNase and protease (Boer, 1975). Protein was measured using the Lowry method (Lowry *et al.*, 1951). Data are presented as mean ± SEM ($n$ = 4–8) for each treatment group; significance was evaluated using analysis of variance and Student's $t$ test.

## RESULTS

Under the culture conditions utilized in this study, the conceptuses of pregnancy Day 11 grew considerably with extensive differentiation of the major organs in the control experiments. The DNA and protein contents in the embryo increased approxi-

mately 10- to 15-fold from the initial Day 11 values after 46–48 hr of culture from 6.1 ± 0.3 and 67.0 ± 3.1 μg/embryo to 115 ± 10.7 and 796 ± 78.2 μg, respectively ($n$ = 12). Increases in the yolk sac DNA and protein contents were also similar with 2.9 ± 0.1 and 54.9 ± 2.0 μg/yolk sac increasing to 38.5 ± 1.2 and 636 ± 33.5 ($n$ = 12). The growth of the allantoic placenta was marginal or absent. The deleterious effects of the two direct alkylating agents, TEM and nitrogen mustard, were marked. At two different doses, 1 and 5 μg/ml, conceptus growth was markedly retarded (Figs. 1A and B). DNA and protein contents of embryos and yolk sacs were significantly reduced compared to controls ($p < 0.001$). The organogenesis of the embryo did not progress.

Cyclophosphamide alone at doses 0.35 mM or microsomes (0.5 mg protein/ml) and NADPH (1 mM) added to the culture medium produced no significant changes in the DNA and protein contents of embryos and yolk sacs of conceptuses when compared to controls. The growth and differentiation of embryos were comparable to the controls. However, conceptus development *in vitro* was markedly affected in the medium containing the same amounts of microsomes, NADPH, and cyclophosphamide added together (Fig. 1C). The yolk sac diameters of these conceptuses were significantly smaller than the controls. The somite numbers of embryos were reduced considerably and frequently somites were not distinguishable. All these embryos were abnormal, and the heart was not beating at the end of the culture period. DNA and protein contents of embryos and yolk sacs were significantly reduced in these experiments ($p < 0.001$).

In similar experiments, aminopyrine in equimolar quantity to cyclophosphamide, either alone in the media or in combination with same amounts of microsomes and NADPH, had no apparent adverse effects on conceptus growth or embryonic differentiation (Fig. 1D). Conceptus growth was comparable to controls.

## DISCUSSION

The present study describes the induction of anomalous embryo development *in vitro* by teratogens acting either directly or indirectly through biotransformation into active metabolites by microsomal enzymes. Aminopyrine, which has not been demonstrated to be teratogenic, did not cause anomalous development in this system. The cytotoxic properties of alkylating agents are due to their strong reactivity toward macromolecules such as nucleic acid and proteins (Connors, 1975); and cytotoxicity to tissues during organogenesis is one of the primary mechanisms of anomalous development (Scott, 1978). In a more elaborate study with TEM utilizing the same techniques, cytotoxicity was found to be distinctly stage specific, i.e., pregnancy Day 11 conceptuses were more severely affected than were those taken on pregnancy Day 12 (M. K. Sanyal and E. El-Nahass, unpublished). In similar studies, Sadler and Kochkar (1976) have shown that chlorambucil inhibited DNA synthesis in embryos from Day 12 pregnant mice even within a short period of 4–8 hr, but RNA and protein synthesis continued.

Experiments in which adult rat hepatic microsomes and NADPH were added to cyclophosphamide in the medium clearly showed that growth and differentiation of embryos were affected in this combined treatment group. It is known that liver microsomes rapidly metabolize cyclophosphamide *in vitro* (Connors *et al.*, 1970, 1974; Cohen and Jao, 1970; Sladek, 1971). In the present study, it seems likely that toxic metabolites of cyclophosphamide began to interfere with the differentiation of cells and tissues as soon as cyclophosphamide, microsomes, and NADPH were added to the culture medium. The embryo, yolk sac, or placental tissues apparently did not contain sufficient mixed-function oxidase enzyme system activity to affect developmental processes of the embryo when cyclophosphamide alone was added to the culture me-

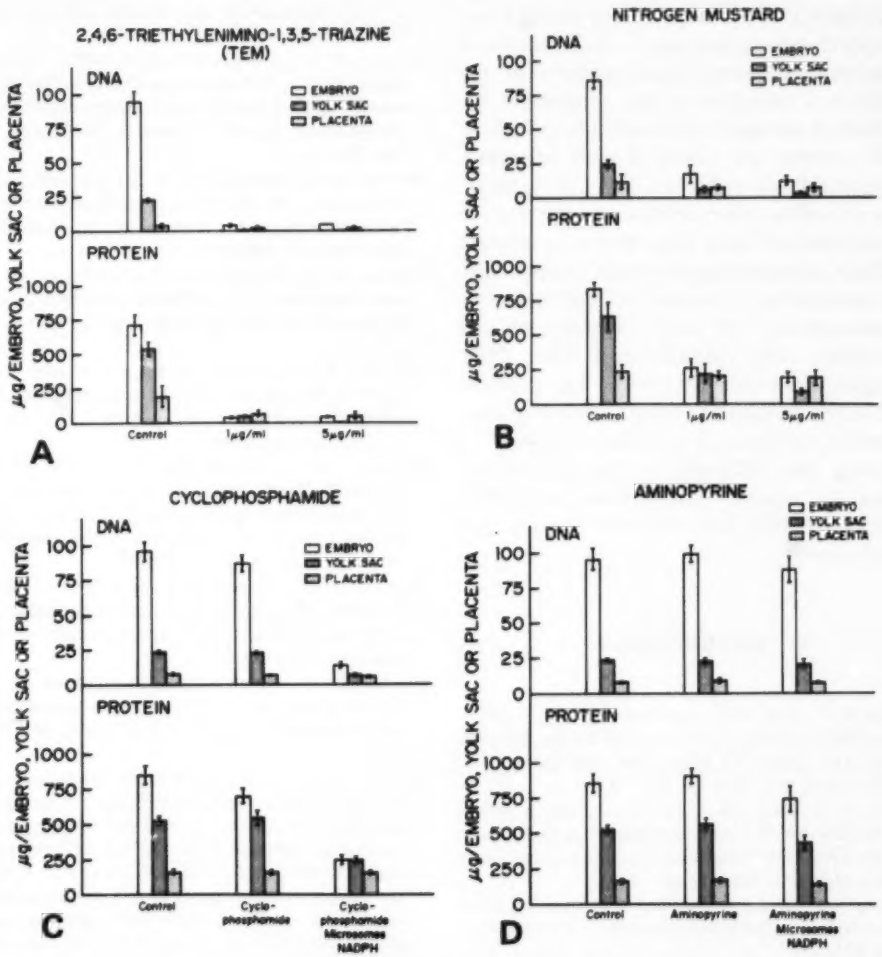


FIG. 1. Effects of teratogens on DNA and protein synthesis of embryos, yolk sacs and placentas of pregnancy Day 11 rat conceptuses cultured *in vitro* for 2 days. (A) TEM at doses 1 and 5 μg/ml medium was highly toxic to embryonic growth; DNA and protein contents of embryos and yolk sacs were reduced significantly ($p < 0.001$) from those of controls. (B) The toxic effects of nitrogen mustard at doses 1 and 5 μg/ml medium were similar to those of TEM with significantly reduced embryo and yolk sac DNA and protein contents ($p < 0.001$). (C) The conceptus growth was unaffected in the presence of cyclophosphamide alone in the culture medium. However, addition of cyclophosphamide (0.35 mM), microsomes (0.5 mg protein/ml), and NADPH (1 mM) to the culture medium induced marked deleterious effects on the conceptus development; DNA and protein contents of embryos and yolk sacs were significantly reduced from control values ($p < 0.001$). (D) Aminopyrine (0.35 mM) alone or a combination of aminopyrine, microsomes (0.5 mg protein/ml), and NADPH in the medium did not affect conceptus development.

dium. The anatomical changes induced by the activated metabolites of cyclophosphamide described by Fantel *et al.* (1979a, b), and also by our studies (Kitchin *et al.*, in press) indicate that activation of the drug is essential for its dysmorphic action.

Coupling of the microsomal mixed-function oxidase enzyme system with culture of conceptuses during organogenesis of the embryo is analogous to the system for detection of mutagens in which tester *Salmonella* strains are cultured with postmitochondrial fraction (Ames *et al.*, 1973, 1975). The mixed-function oxidase enzyme system in microsomes have been shown to activate indirect acting mutagens in this system. Similar approaches have been utilized in several *in vitro* studies with human lymphocytes and fibroblast cells (Mandle and Obe, 1978; Cooper and Goldstein, 1976). The possible use of the conceptus culture system in conjunction with *in vivo* tests for evaluation of directly and indirectly acting teratogens, however, requires further study with other agents of different chemical nature and mechanism.

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